DEVELOPMENT AND EVALUATION OF NEWER POLYHERBAL ANTIMICROBIAL AND ANTIFUNGAL FORMULATION

A

SYNOPSIS

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ANNEXURE II

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INTRODUCTION

India is one of the world's leading biodiversity centers with the presence of over 45000 different plant species. India has 15000 – 18000 species of flowering plants, 2500 algae, 23000 fungi, 1600 types of lichens and 1800 varieties of bryophytes and estimated 30 million types of microorganisms. Out of these about 15000 – 20000 plants have good medicinal value. However only traditional communities use about 7000-7500 plants for their medicinal values. Plant chemistry has been the basis for many synthetic drugs in modern Pharmacopoeia.[1] Plant products have been part of phytomedicines and herbo-cosmeceutical products since time immemorial. These can be derived from any part of the plant like bark, leaves, flowers, roots, fruits and seeds i.e., any part of the plant may contain active components[2]. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances. Such Phytochemical screening of various plants is reported by many workers[3]. The Indian subcontinent is enriched by a variety of flora- both aromatic and medicinal plants. Herbal drugs constitute a major part in all the traditional systems of medicine. Now a days there are so many herbal plants used as ingredients in many skin care preparations, e.g., Calendula officinalis as an emollient, Hemamelis virginiana as an astringent, Arctium lappa as a depurative (tighten the skin), Prunella vulgaris and Symphytum officinalis, aid the healing of cuts, wounds and abrasion. Throughout history and in most of civilizations, scalp hair has been associated with positive signals such as beauty and power. Baldness or hair loss on the other hand has a negative attributo[4].

In general, 50-100 hairs at random are shed everyday. Various factors contribute to hair fall / loss. Genetic predisposition and hormonal factors predominantly contribute to the above. Diseases state such as typhoid, malaria and jaundice also cause hair fall. The use of chemotherapeutic agents also causes hair fall. Management of hair fall is extremely complex. Hormone therapy, use of α-reductase inhibitors, vasodilators like minoxidil is widely used to reduce the hair fall / loss. The use of some of the herbal oils is also reported to reduce the hair fall / loss. In ancient Greece and Rome, countless ointments and tonics were recommended for the beautification of the hair, as well as remedies for the treatment of scalp diseases. Dandruff is a common scalp disorder affecting almost half of the post pubertal population of any ethnicity and both genders. The exact nature and etiology of dandruff has always been controversial since the time of the Greeks, through Sabouraud's era in late nineteenth century till to-date. Dandruff

represents 25 % of all scalp disorders. It is present in an estimated 15-20 % of the total population and more than 50 % of adult population[5].

Alopecia is dermatological disorder that has been recognized for more than 2000 years and a common problem in cosmetics as well as primary health care practice. It is common throughout the world and has been estimated to affect between 0.2 % and 2 % of the world population. Androgens are considered to be one of the most important causes for alopecia apart from a variety of other factors[6]. Synthetic drug, Minoxidil is a potent vasodilator was scientifically proved for the treatment of alopecia. Though the use of drugs for its side effect is not advisable, the drug of plant origin is necessary to replace the synthetic one. So in the proposed work, an attempt has been made to prepare and characterize some selected herbal formulations containing antimicrobial and antifungal plants. It has also been planned to evaluate prepared formulations in animals through preclinical studies.

NEED OF STUDY

India has rich tradition of plant based knowledge of healthcare. The use of the plant based medication is gradually becoming popular throughout the world. The traditional Indian systems of Ayurveda and Siddha medicines support the importance of medicinal plants to treat diseases. The Director of WHO Traditional Medicine reported in 1993 that 80% of the world populations rely chiefly on traditional medicine, mainly plant based, specially for their primary health care needs. In India 70% of populations are reported using traditional medicine for primary health care. The present annual turnover of herbal medicinal products manufactured by large companies is estimated to be approximately US $ 300 million, compared to a turnover of approximately US $ 2.5 billion for modern drugs. Herbal medicine has become an item of global importance both medicinal and economical. World Health Organization (WHO) has defined herbal medicines are finished, labeled medicinal products that contain active ingredients, aerial or underground parts of the plants or other plant material or combination.

Although usage of these herbal medicines has increased, their quality, safety and efficiency are serious concerns in industrialized and developing countries. Herbal remedies are getting increasing patient compliance as they are devoid of typical side effects of allopathic medicines.

The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body to promptly achieve and then maintain the desired drug concentrations. The route of administration has a significant impact on the therapeutic outcome of a drug. Skin is one of the most readily accessible organs on human body for topical administration and is main route of topical drug delivery system. Topical delivery can be defined as the application of a drug containing formulation to the skin to directly treat cutaneous disorders (e.g. acne) or the cutaneous manifestations of a general disease (e.g. psoriasis) with the intent of containing the pharmacological or other effect of the drug to the surface of the skin or within the skin. Semi-solid formulation in all their diversity dominate the system for topical delivery, but foams, spray, medicated powders, solutions, as well as medicated adhesive systems are also in use. So in the proposed work, an attempt has been made to prepare and characterize some selected herbal formulations containing antimicrobial and antifungal plants. It has also been planned to evaluate prepared formulations in animals through preclinical studies.

OBJECTIVES OF THE STUDY

1. To Collect, identify and study quality parameters for authentication of the crude drugs.
2. To Prepare, optimize and chemical analysis of obtained extracts.
3. To Develop TLC fingerprinting of the extracts.
4. To investigate the anti-fungal and antibacterial activity of extracts.
5. To prepare herbal formulations with the use of several semisolid hair care bases to incorporate the selected antibacterial, antifungal herbal extracts.
6. To study physicochemical parameters of prepared herbal formulations.
7. To evaluate prepared herbal formulations for antifungal and antibacterial activity.
8. To study prepared herbal formulations on dermal irritancy in rabbits and hair growth promoting activity in mice.

MATERIALS AND METHODS

In the present work the basic materials required are an anti-microbial and antifungal herbal plants like *Hibiscus abelmoschus, Pterocarpus marsupium, Ranunculus sceleratus Linn. Cassia fistula, Momordica charantia and Calendula officinalis.* Etc.

- Culure media (Sabourand's and sabouraud's dextrose agar).
- Beeswax, carnauba wax, paraffin wax, mineral and vegetable oils, petroleum jelly, spermaceti, lanolin anhydrous, isopropyl myristate, stearic acid, almond oil, sandal wood oil, coconut oil etc.
- Antioxidants like Butilated hydroxy toluene, Butilated hydroxyl anisole etc and preservatives like methyl and propyl parabens.

It has been planned to formulate and characterize the formulations in several phases.

Phase-I: - Plant materials will be collected from appropriate places followed by their authentification from botanist.

Phase-II: - All the selected plants will be subjected to the successive extraction by using different organic solvents then it is planned to analysis of all the selected extract by using various analytical techniques for their physicochemical properties by TLC fingerprinting, HPTLC and Spectroscopic methods etc.).

Phase-III: -

The selected extracts will be subjected for antimicrobial and antifungal screening by using suitable culture media.

Phase-IV: - Preparation of Polyherbal Gel

The selected extracts for the better antifungal and antimicrobial action will be suitably incorporated in suitable bases using reported and conventional methods.

Phase-V: - Evaluation Polyherbal topical gel

1. Physical Examination

2 pH determination

3 Spreadability

4. Viscosity measurements and rheological behavior

5. In Vitro drug release kinetics:

Mice Skin for the diffusion studies:

Mice skin was washed in the running water. It was then soaked in distilled water for24 hours, before used for diffusion studies to remove glycerin present on it and was mounted on the diffusion cell for further studies.

Procedure:

The in vitro diffusion studies of prepared gel were carried out on Mice skin. 100 ml of phosphate buffer of pH 7.4was used as receptor compartment, and then 1g of gel was spread skin facing the donor compartment. The donor compartmentwas kept in contact with a receptor compartment and the temperature was maintained at37±100C. The buffer solution was kept on the receptor side. At pre determined time intervals, pipette out 5ml of solution from the receptor compartment and immediately replaced with the fresh 5ml phosphate buffer. The drug concentration on the receptor fluid was determined spectrophotometrically.

7. Stability studies.

Optimized formulation was selected and kept for stability studies. Formulations were packed in a glass bottles and studies were carried out for 30 days by keeping at 35^0C,40^0C.

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Phase-VI: - Evaluation for antifungal and antimicrobial actions of the Polyherbal Gel

Herbal formulations will be subjected for screening of antimicrobial and antifungal action by compering with standard antimicrobial and antifungal drugs.

Phase-VII: - Pre-clinical studies

Skin Irritation study on rabbits:

To test the irritancy potential of experimental Topical gel rabbits were used; the selected Gels were applied to the shaved skin on the rabbits back. The selection of rabbits for this Study is described as follows.

Test animal – Healthy Rabbits

Strain – New Zealand white rabbits

Age – 22 weeks

Sex – Male

Weight – 2-2.5 kg

No of animals per group – 3

Selection and Randomization:

Animals were randomly placed in cages upon receipt and then randomized according to the body weights, Animals considered unsuitable because of outlaying body weights were excluded from the study. The animal backs were carefully shaved using sterilized shaving blade to remove the fur. The selected animals were grouped into 4 groups. and were marked with identification codes using diluted picric acid solution (1.2%w/v). Circular areas of 2.54 cm (1 inch diameter) were marked on the back of each animal using marker ink one spot on right side and one spot on left side of vertebral column.

The spot on right side was abraded using shaving blade and the spot on left side was kept intact 1gm quantity of the selected gels was applied on marked spots using absorbent cotton wool. The toxic manifestations if any on the skin were then assessed by observing the skin at pre-selected time intervals after treatment e.g. 1 hrs, 4 hrs, 12 hrs, 24 hrs, 48 hrs and 72 hours,

IN VIVO EVALUATION for Anti-fungal activity

Animals: Adult Wistar rats (280 ± 10 g) of either gender The animals will be housed in large, spacious polyacrylic cages at an ambient room temperature with 12-h light/12-h dark cycle. Rats had free access to water and rodent pellets diet. The study will be carried as per CPCSEA guidelines.

Acute toxicity testing: The female rats will be used for the acute toxicity testing. Hair present in the dorsal surface of the animal (2 X 2 cm) will be removed by applying hair remover and cleaned with alcohol. The screening will be marked (1 X 1 cm) and 0.5 g of polyhedral gel will be applied to the surface of an animal's skin. During the observation period (14 days), signs such as erythema and edema were assessed.

Evaluation of therapeutic efficacy: Healthy, adult, male Wistar rats will be used for the experiment. The rats were divided into the four groups each of six animals viz., normal control (group I), fungal control (group II), standard treatment group (group III) and Polyherbal gel treatment group (group IV). Group II to IV animals will be changed with intravenous methylprednisolone (5mg/kg) for three days for induction and maintenance of cell mediated immunosuppression (Organisms from stock isolates were stored in nutrient agar at 27°C, streaked onto nutrient broth, and incubated at 37°C for 24 h and included culture was used for further experiment). Fungal culture diluted with PBS and swabbed in smooth muscle of rat pennies and allowed to grow for 3 days until the growth of Fungi observed on ischiocavernosus smooth muscle. The colony growth was confirmed by counting colony-forming-unit. The animals which as cfu value of more than 3 cfu/ml will be included in the study. The animals will be treated for week period and visually observed its physical changes. The swab culture was collected on initial day, 4th and 7th day of the experiment for microscopical evaluation. End of the experiment the animals will be sacrificed and ischiocavernosus smooth muscle was collected from all the experimental animals and preserved in 10% formalin. Microscopical evaluation: The colony was collected in sterile cotton swab and transferred into 0.5 ml sterile phosphate buffer saline (PBS). The mixture was diluted 10 fold and inculcated in nutrient agar media, incubated for 48 h at 37°C. The yeast count was expressed as log 10 of cfu/ml of PBS.

Microscopical evaluation: The colony will be collected in sterile cotton swab and transferred into 0.5 ml sterile phosphate buffer saline (PBS). The mixture was diluted 10 fold and inculcated in nutrient agar media, incubated for 48 h at 37°C. The yeast count will be expressed as log 10 of cfu/ml of PBS.

Histopathologic analysis: The rat penal smooth muscle will be dehydrated with alcohol for 12 h each and cleaned with xylene for 15-20 min. The tissue blocks were prepared and the blocks were cut using microtome to get sections of thickness 5 μm. The sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied and allowed for drying. Finally, the sections will be stained with eosin (acidic stain) and hemotoxylin (basic stain)

Statistical analysis:

All the data will be analyzed by One-way analysis of variance (ANOVA)

REVIEW OF LITERATURE:-

In **Pushpendra Kumar Jain et al**, study, where extracts are prepared by separating the soluble matter from vegetable tissues by the application of a suitable solvent such as alcohol, water, or ether. The resultant liquid is concentrated by evaporation to obtain a liquid extract or concentrated almost to dryness to obtain the solid extract and its volatile oil. Hair formulation of *C. rotundus* belonging to family *Cyperaceae* in the form of herbal formulation (5% herbal cream and oil) was studied and it showed excellent hair growth activity with standard (2% minoxidil ethanolic solution) in Wister albino rats[7].

In **M. Santhosh Aruna et al** study, where prepared hair gel formulation which showed optimal release was subjected to antifungal activity by adopting disc diffusion method. The test organizing was Pityrodporium Ovale (strain 27) in sabouraud's dextrose agar media. Commercial Clotrimazole ointment was taken as standard. Clotrimazole is a well-known effective antifungal drug and it is available as a topical formulation. The formulated herbal gel did not show any irritation and erythema after 48 hours. This indicates better skin acceptability of herbal gel formulation. The formulations showed better release and maximum zone of inhibition than marketed formulation. Hence, Hair gel formulation prepared from herbal was considered as best formulation[8].

In **Roopashree TS et al**., study the present investigation was carried out to study the unexplored area of these drugs towards their antibacterial activity with respect to their traditional use as antipsoriatic agents. The herbs were subjected to successive extraction using different

solvents and the extracts were subjected to antibacterial evaluation against both gram positive and gram negative organisms by cup plate technique. Among the various extracts, aqueous extracts were found to be more effective against all the bacteria. Staphyllococcus aureus was more susceptible to the aqueous extracts among the tested organisms. Minimum inhibitory concentrations of different extracts against the tested organisms are as follows: Momordica charantia extracts exhibited MIC ranging from 50 μg/ml to 32 mg/ml (Table-3) against the tested organisms, except ethanolic extract which did not inhibit the growth of B. subtilis and E. coli at any of the concentrations used in the study. In case of Calendula officinalis, ethanolic and aqueous extracts inhibited the growth of the bacteria used in the study at concentrations ranging from 125μg/ml to 64mg/ml. Methanolic extract inhibited the growth of both S. aureus and E.coli at 64mg/ml; whereas none of the bacteria were inhibited by the petroleum ether extracts of Calendula officinalis atany of the concentrations tested. Aqueous extract of Cassia tora inhibited S. aureus, P. aeruginosa and E. coli at concentrations of 100μg/ml, 200μg/ml and 250μg/ml respectively but did not inhibit the growth of B. subtilis at any of the concentrations tested. Ethanolic extract inhibited only B. subtilis, where as it was not effective against the other bacteria tested. Methanolic extract was effective against two of the tested organisms i.e., S. aureus and E. coli both at the concentration of 64mg/ml. Pet ether did not inhibit the growth of any of the bacteria[9].

In **T Regupathi etal** study, Ethyl acetate soluble fraction of ethanolic extract of Eclipta alba and Lippia nodiflora was evaluated for its potential on hair growth by in vivo method. In vivo, 5 % and 10 % extract of E. alba and L. nodiflora alone and also in combination of 5 % of E. alba and 5 % of L. nodiflora in gel formulation was applied topically over the shaved skin of black mice and monitored and assessed for 30 days. The length of hair and the different cyclic phases of hair follicles like anagen and telogen phases were determined at different time periods. From the study, topical use of gel formulations containing extracts of E. alba and L. nodiflora alone and also in combination onto the shaved area on the animals, produced a significant increase in the rate of hair growth compared with negative control (simple gel alone). It is concluded that the ethyl acetate soluble fraction of ethanolic extract of E. alba (5 %) and L. nodiflora (5 %) combination gel formulation exhibits more potency on hair growth activity. the effect of EEEA and EELN gel formulation on the qualitative hair growth and length was found to be more significant as compared to standard and control group treated animals. The

quantitative effect of HG5 gel formulation definitely promotes hair growth by inducing hair follicles in the anagen phase. The percentage of anagen induction with EEEA 5 % + EELN 5 % (HG5) gel formulation and minoxidil were comparable. On the basis of similarities observed between the minoxidil and the HG5 studies, it is expected that EEEA 5 % + EELN 5% (HG5) gel formulation showed the best hair growth activity compared with minoxidil. In future this gel formulation will be tested on human volunteers for its hair growth activity[10].

In **Luis J et a**l., study Seborrheic Dermatitis (SD) and dandruff are of a continuous spectrum of the same disease that affects the seborrheic areas of the body. Dandruff is restricted to the scalp, and involves itchy, flaking skin without visible inflammation. SD can affect the scalp as well as other seborrheic areas, and involves itchy and flaking or scaling skin, inflammation and pruritus. Various intrinsic and environmental factors, such as sebaceous secretions, skin surface fungal colonization, individual susceptibility, and interactions between these factors, all contribute to the pathogenesis of SD and dandruff. In this review, we summarize the current knowledge on SD and dandruff, including epidemiology, burden of disease, clinical presentations and diagnosis, treatment, genetic studies in humans and animal models, and predisposing factors. Genetic and biochemical studies and investigations in animal models provide further insight on the pathophysiology and strategies for better treatment. Various intrinsic and environmental factors, such as Malassezia yeast, host epidermal conditions, sebaceous secretion, immune response, and the interactions between these factors, may all contribute to the pathogenesis. Effective management of SD and dandruff requires clearing of symptoms with antifungal and anti-inflammatory treatment, ameliorating associated symptoms such as pruritus, and general scalp and skin health to help maintain remission. Studies in humans and animal models to investigate the genetic and biochemical pathways will help identify new targets for the development of more efficacious treatment with less side effects, and better management of these conditions[11].

In **Abhishek Dwivedi** study**,** *Abelmoschus moschatus* Medik. is an aromatic and medicinal plant in the Malvaceae family, which is native to India. It is an erect hispid herbaceous trailing herb that grows up to 1.5m tall with a long slender tap root. Leaves are alternate, rough, hairy and heart-shaped. They have 3 to 5 lobes and can grow to 15cm long. Flowers resemble those of the hibiscus and are usually watermelon pink, although they are sometimes white or cream in colour. They last for only one day and their flowering depends on the timing of the wet

season. Seeds are contained within hairy capsules up to 8 cm long, which are tough but papery. The seeds have a sweet, flowery, heavy fragrance similar to that of musk. The present paper deals with review on pharmacological and phytochemical profile of *Abelmoschus moschatus*[12].

In Jaya Raja Kumar study, The goal of this study was to create and characterize itraconazole (ITZ) ethosomal gel which may serve as a more efficient agent in local drug deliveryas compared to the current gel preparation available in the market. On the other hand, itraconazole ethosomal gel is fabricated with an aimto solve the limitationsof oral delivery of drugs. The formulations were prepared with varying the quantity of cholesterol (100, 125, 150 and 175 mg), static volume of dichloromethane (DCM), ethanol, phosphate buffer and polaxamer 188.The formulations were evaluated for viscosity, gel strength, spreadability, invitro release, in-vivo therapeutic efficacy, microscopic analysis histopathological analysis and Fourier Transformed Infrared (FTIR) studies.[13]

PLAN OF WORK:

Different phases of Research work planned are as follows:-

a) Literature survey

b) Collection of plants and authentication

c) Preliminary phytochemical screening

d) Extraction of plants by using organic solvent

e) Antimicrobial studies of different extracts

f) Formulation development and evaluation

g) Preclinical studies

DURATION OF WORK:

Sr. No.	Work to be Completed	Duration
1	Review of Literature	4 Months
2	Procurement of plants and authentication	2 Months
3	Preliminary phytochemical screening	4 Months
4	Extraction of pants by using organic solvent	4 Months
5	Antimicrobial and antifungal studies of obtained extracts	8 Months
6	Development and Evaluation of herbal formulation	8 months
7	Preclinical studies	6 Months
Total Duration of Project		**36 Months**

BIBLIOGRAPHY:

1. Marjorie Murphy Cowan, Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 1999, 12(4):564-82.
2. Gordon M. Cragg & David J. Newman. Natural Product Drug Discovery in the Next Millennium. Pharmaceutical Biology 2001, Vol. 39, Supplement, pp. 8–17.
3. Faraz Mojab, Mohammad Kamalinejad, Naysaneh Ghaderi, Hamid Reza Vahidipour, Phytochemical Screening of Some Species of Iranian Plants. Iranian Journal of Pharmaceutical Research 2003; 2: 77-82.
4. Jigna Parekh, and Sumitra Chanda, Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. African Journal of Biomedical Research, 2007; 10: 175 – 181.
5. Herrera-Arellanoa, S. Flores-Romerob, M.A. Chavez-Sotoc, J. Tortorielloa, Effectiveness and tolerability of a standardized extract from Hibiscus sabdariffa in

patients with mild to moderate hypertension: a controlled and randomized clinical trial. Phytomedicine 2004; 11: 375‑382.

6. Bagatell CJ, Heiman JR, Matsumoto AM, Rivier JE, Bremner WJ. Metabolic and behaviour effects of high dose exogenous testosterone (T) in normal men. J Clin Endocrinol Metab 1994; 79: 561-7.
7. Pushpendra Kumar Jain, Debajyoti Das, Singhai AK, Alternative Herbal Drugs Used For Treating Hair Disease. Asian J Pharm Clin Res, Vol 9, Issue 1, 2016, 110-112.
8. M. Santhosh Aruna, N. Sai Amani, P. Sri Lakshmi, A. Sravani, M. Surya Prabha, N. Ramarao, Formulation and Evaluation of Herbal Anti Dandruff Gel. Indo American Journal of Pharm Research.2014; 4(11): 5389-93.
9. Roopashree TS*1, Raman Dang 1, Shobha Rani RH1, Narendra C2, Antibacterial activity of antipsoriatic herbs: Cassia tora, Momordica charantia and Calendula officinalisI international Journal of Applied Research in Natural Products 2008;1(3); 20-28, Sep/Oct
10. T Regupathi1*, K Chitra2, K Ruckmani3, KG Lalitha4 and Mohan Kumar, Formulation and Evaluation of Herbal Hair Gel for Hair Growth Potential, J of Pharmacol & Clin Res 2017; 2(2): 555-581.
11. Luis J. Borda and Tongyu C. Wikramanayake, Seborrheic Dermatitis and Dandruff: A Comprehensive Review, J Clin Investig Dermatol. 2015 December
12. Abhishek Dwivedi1* and Ameeta Argal, A Review on Pharmacological and Phytochemical profile of Abelmoschus moschatus Medik, CODEN (USA): IJPLCP, July, 2015:4657-4660.
13. Jaya Raja Kumar, Development and Antifugal Activity of Itraconazole Loaded Ethosomal Gel in Rat Animal Model, /J. Pharm. Sci. & Res. Vol. 11(7), 2019, 2728-2733

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